References Cited [Referenced By]

U.S. Patent Documents

5861484	Jan., 1999	Kendall et al.	530/350.

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Parent Case Text

This application is the national stage of PCT/GB95/01213, filed May 26, 1995.

Claims

We claim:

1. An isolated, soluble polypeptide selected from the group consisting of:

(a) a polypeptide having an amino acid sequence consisting of amino acid residues 1 to 553 of SEQ ID NO:3;

(b) a polypeptide having an amino acid sequence consisting of amino acid residues 1 to 517 of SEQ ID NO:3;

(c) a polypeptide having an amino acid sequence consisting of amino acid residues 1 to 553 of SEQ ID NO:3 with the addition of the sequence ELSNFECLHPCSQE (SEQ ID NO:16) at its C-terminal end;

(d) a polypeptide having an amino acid sequence consisting of amino acid residues 1 to 517 of SEQ ID NO:3 with the addition of the sequence LPPADSSFMLPPTSFSSNYFHFLP (SEQ ID NO:17) at its C-terminal end; and

(e) a truncated variant polypeptide of (a), (b), (c) or (d); wherein said truncated variant polypeptide consists of no more than five complete immunoglobulin-like domains; and wherin said trucated variant polypeptide both binds to and exerts an inhibitory effect on VEGF.

2. The isolated soluble polypeptide of claim 1 which is a truncated variant polypeptide that consists of no more than four complete immunoglobulin-like domains.

3. The isolated soluble polypeptide of claim 1 which is a truncated variant polypeptide that consists of from about 400 to about 500 amino acid residues.

4. An isolated nucleic acid having a nucleotide sequence that encodes the isolated soluble polypeptide of claim 1 part (a),(b),(c) or (d), or claim 3.

5. A method for inhibiting VEGF in vitro comprising adding an effective amount of the isolated soluble polypeptide of claims 1, 2 or 3.

6. A pharmaceutical composition comprising the isolated soluble polypeptide of claims 1, 2 or 3, and a physiologically acceptable carrier.

Description

FIELD OF THE INVENTION

This invention relates to substances which inhibit growth factors, in particular, vascular endothelial growth factor (VEGF), methods of inhibiting growth factors and of treating tumours and regulating fertility.

BACKGROUND OF THE INVENTION

A considerable number of human growth factors are now known, many of which have been at least partly characterised. Among them is vascular endothelial growth factor (VEGF), which has been identified in several tissues (Gospodarowicz et al., 1989 PNAS 86, 7311-7315; Conn et al., 1990 PNAS 87, 2628-2632; Tischer et al., 1991 J. Biol. Chem. 266, 11947-11954). As its name suggests, this growth factor is a highly specific mitogen for endothelial cells and is greatly involved in angiogenesis. VEGF is a homodimeric glycoprotein of two 23 kDa subunits exhibiting sequence homology with platelet-derived growth factor A and B chains and placenta growth factor.

The homologous tyrosine kinase receptors fms-like tyrosine kinase receptor (FLT) and kinase insert domain-containing receptor (KDR) function as high-affinity VEGF receptors (de Vries et al., 1992 Science 255, 989-991; Terman et al., 1992 Biochem. Biophys. Res. Commun. 187, 1579-1586). Both FLT and KDR are membrane-spanning receptors that each contain seven immunoglobulin-like domains in the extracellular ligand-binding region, an intracellular tyrosine kinase domain and a transmembrane domain. The transmembrane domain serves to anchor the receptor in the cell membrane of the cells in which it is expressed.

A number of membrane-bound receptor molecules have been found to exist in truncated soluble forms, generated either by proteolytic processing or by alternative splicing of mRNA. Recently, Kendall & Thomas (1993 PNAS 90, 10,705-10,709, and WO94/21679) described the discovery of a soluble form of FLT receptor (sFLT) generated by alternative splicing.

Essentially, Kendall & Thomas screened a human umbilical vein endothelial cell (HUVEC) cDNA library with one probe specific for the 3' end of the fit coding region (encoding the intracellular tyrosine kinase domain) and with another probe specific for the 5' fit coding portion (encoding one of the extracellular N terminal domains). Clones which hybridised with the 5' specific probe but not with the 3' specific probe were selected for further study. In this way, a clone was isolated which encoded a soluble FLT polypeptide lacking the transmembrane domain and the intracellular domain. The truncation resulted from "readthrough" to an intronic termination codon. It was suggested by Kendall & Thomas that the soluble receptor could act as an efficient specific antagonist of VEGF in vivo.

The present invention is based on the discovery of further soluble variants of FLT, the existence of which was not predicted by the teaching of Kendall & Thomas.

SUMMARY OF THE INVENTION

In a first aspect the invention provides an altered, soluble form of the FLT polypeptide being capable of binding

to VEGF and therebv exerting an inhibitory effect thereon, the polypeptide comprising five or fewer complete immunoglobulin-like domains. Preferably, the altered FLT polypeptide comprises four or fewer complete Ig-like domains. The altered soluble FLT polypeptide inhibits VEGF by preventing it binding to its natural receptors, fit and KDR, present on the surface of target cells. Surprisingly, such truncated forms, lacking a major extracellular portion of the molecule, are believed to retain affinity for VEGF.

The term "soluble" as used herein is intended to refer to altered forms of the FLT polypeptide which do not comprise a transmembrane domain and thus generally do not become associated with the cell membrane of cells in which the molecule is expressed. In particular, the invention provides soluble altered forms of the FLT polypeptide consisting substantially of four or five complete immunoglobulin-like domains.

In a particular embodiment the invention provides an altered, soluble form of FLT having at its C-terminus a region substantially having the amino acid sequence of the sequences termed FLT4 or FLT15 shown in FIG. 5, or a functional equivalent thereof. The term "functional equivalent" as used above is intended to include those polypeptides which have substantially the same deletions as the polypeptides encoded by FLT4 (SEQ ID NO:8) or FLT15 (SEQ ID NO:9) (with respect to the unaltered full length FLT molecule), but which may also have other deletions, additions or substitutions, (in particular conservative substitutions), and which retain an inhibitory effect for VEGF.

Preferably the polypeptide will also comprise, at its N-terminus, the amino acid sequence substantially corresponding to the equivalent portion of the unaltered wild-type FLT polypeptide. Conveniently, polypeptides in accordance with the invention will comprise around 400 to 500 amino acid residues, preferably around 480 amino acid residues, most preferably between 480 and 440 amino acid residues of the wild type FLT sequence. Preferably the polypeptides of the invention arise by alternative splicing of mRNA or by proteolytic processing of a mature polypeptide, although it will be apparent to those skilled in the art that the polypeptide could be encoded by a nucleic acid derived, at least in part, by recombinant DNA technology.

In a further aspect the invention provides a nucleic acid sequence encoding a polypeptide in accordance with the invention. In a particular embodiment the invention provides a nucleic acid comprising the sequence of nucleotides inserted at position 1655 of the FLT 4 sequence shown in FIG. 3 or the sequence of nucleotides inserted at position 1555 of the FLT 15 sequence shown in FIG. 3, or a functional equivalent thereof. Examples of functionally equivalent nucleic acids include those sequences which encode substantially the same polypeptide as those encoded by FLT4 or FLT15 but which differ in nucleotide sequence as a result of the degeneracy of the genetic code. It will be apparent to those skilled in the art that the portion of the inserted nucleotide sequence in FLT4 and FLT15 occurring after the premature termination codon could be omitted without affecting the characteristics of the encoded polypeptide. Accordingly, nucleic acid molecules without such sequences are also regarded as functionally equivalent for the purposes of the present invention.

Conveniently, the nucleic acid will substantially comprise the nucleotide sequence of FLT4 or FLT15 shown in FIG. 3, together with the nucleotide sequence encoding the N-terminus of unaltered, wild-type FLT. Advantageously, the nucleic acid will be obtainable by means of PCR amplification from a sample of human cells. Desirably, the nucleic acid will be obtainable by means of PCR using primers intended to hybridise to non-conserved regions of the FLT molecule. Conveniently, the nucleic acid sequence will be obtainable by use of PCR primers designed to hybridise to the regions of the FLT sequence shown underlined in FIG. 3. or immediately adjacent thereto. In particular, the PCR primers will conveniently have substantially the sequence: 5'-GCAAGGTGTGACTTTGTTC-3' (SEQ ID NO:10) and 5'-AGGATTTCTTCCCCTGTGTA-3' (SEQ ID NO:11).

In another aspect, the invention provides a method of inhibiting VEGF in vitro, comprising adding an effective

amount of the polypeptide defined above. It may also be desirable to inhibit VEGF in a human subject. Thus the invention provides a method of inhibitng VEGF in a human subject, comprising administering an effective amount of the polypeptide defined above, together with a physiologically acceptable carrier substance. In particular, VEGF provides a mitogenic stimulus (particularly involved in angiogenesis), so inhibition of VEGF would be expected to provide therapeutic effects in the treatment of tumours or disorders involving inappropriate neovascularisation.

In particular the invention provides for a method of treating tumours or diseases involving inappropriate neovascularisation, comprising administering an effective amount of the polypeptide defined above, together with a physiologically acceptable carrier substance. Suitable diseases which might be amenable to treatment include ovarian cancer and ovarian hyperstimulation (Boocock et al., 1995 J. Natl. Cancer Inst. 87, 506-516).

Furthermore, it has been conclusively demonstrated that FLT is expressed by trophoblasts and cells from ovarian and endometrial tissues (Charnock-Jones et al., 1994 Biology of Reproduction 51, 524-530), which clearly suggests a role for VEGF in the growth and differentiation of trophoblasts during implantation.

Thus, in particular, the invention provides a method of affecting the growth and/or migration of trophoblasts, ovarian or endometrial cells by inhibiting the action of VEGF, comprising administering an effective amount of the polypeptide defined above, together with a physiologically acceptable carrier substance.

It will be appreciated by those skilled in the art that the identification of FLT on the surface of trophoblasts and endometrial cells also provides a number of possible methods of regulating fertility. For example, the growth of trophoblasts is essential for successful implantation of the embryo. Inhibition of trophoblast growth thus provides a method of contraception or contragestion.

Thus in a further aspect the invention provides a method of regulating the fertility of a human female, comprising administering an effective amount of the polypeptide defined above, together with a physiologically acceptable carrier substance. An "effective amount" of the polypeptide is an amount sufficient to substantially block the stimulus of VEGF on trophoblasts and/or endometrial cells. Typically, the method will result in reducing the fertility of the female.

Moreover, it might be possible to identify agents which can enhance the effect of VEGF on trophoblasts, and thereby improve the probability of successful implantation, either in assisted or spontaneous cycles. Candidates for such VEGF-enhancing agents would include anti-sense equivalents of the nucleic acid sequences encoding the truncated FLT polypeptides of the invention. It will be apparent to those skilled in the art that these could be used to improve the fertility of a human female.

In a further aspect the invention provides a pharmaceutical composition comprising the polypeptide defined above, together with a physiologically acceptable carrier substance. The composition could be used in vivo any one of the methods defined above. In yet another aspect the invention provides for the use of a polypeptide in accordance with the invention in the preparation of a therapeutic composition for the treatment of tumours and diseases involving inappropriate neovascularisation. Examples of such conditions and diseases are detailed, inter alia, in WO94/10202 and WO94/21679. The invention also includes within its scope a method of making a pharmaceutical compostion, comprising mixing the polypeptide defined above together with a physiologically acceptable carrier substance.

The invention will now be described by way of the following illustrative examples and with reference to the drawings.

DESCRIPTION OF THE DRAWINGS

FIG. 1 shows an amino acid multiple alignment of closely related tyrosine kinase receptors (flt (SEQ ID NO:3), fms (SEQ ID NO:2) and kit (SEQ ID NO:1), "kit" being another name for KDR);

FIG. 2 shows typical results of agarose gel electrophoresis demonstrating the existence of alternatively-spliced fit-coding sequences in various tissue samples;

FIG. 3 shows the nucleotide sequence of the 3' region of the sequences encoding full length VEGF receptors (FLT (SEQ ID NO:5) and the related receptor KDR (SEQ ID NO:4)), together with two sequences, FLT4 (SEQ ID NO:6) and FLT15 (SEQ ID NO:7), which encode polypeptides according to the invention;

FIG. 4 is a schematic representation of wild type and mutant FLT molecules; and

FIG. 5 shows the C terminal amino acid sequences of two polypeptides (FLT4, SEQ ID NO:8, and FLT15, SEQ ID NO:9 respectively) in accordance with the invention.

EXAMPLE

Expression of FLT, the VEGF receptor, was investigated in cell lines derived from human trophoblast-like and ovarian and endometrial carcinomas. The trophoblast-like (choriocarcinoma) cell line used was BeWo (obtained from the American Type Culture Collection, Rockville Md., USA). The endometrial carcinoma cell lines were Ishikawa (obtained from Professor M Nishide, University of Tsukuba, Japan), and HEC 1-A and HEC 1-B (from ATCC, USA). The ovarian cancer cell lines were 7, 17R, 25, 25R and 35. These were all shown to be of epithelial origin and had been established in culture for 10-30 passages. Cell lines 17R and 25R were derived after chemotherapy and subsequent relapse (line 25R originating from the same patient as line 25).

BeWo cells were grown in Ham's F12, according to ATCC recommendations. Endometrial carcinoma lines were grown in McCoy's medium (ICN Flow Laboratories, Irvine, UK) with 10% foetal calf serum (ICN Flow) plus 2 mM L-glutamine (ICN Flow) and 50 U/ml and 50 mg/ml penicillin/streptomycin (ICN Flow).

It was decided to investigate expression of FLT in these cell lines and normal tissues by performing PCT and in situ hybridization. It was therefore necessary to construct suitable oligonucleotide primers and probes.

To help design appropriate primers, a protein multiple alignment of closely related tyrosine kinase receptors (FLT, FMS and KIT) was constructed (shown in FIG. 1) using the computer program "pileup". This revealed regions of divergent sequence among this family of receptors. The regions chosen for primer design are shown with double underlining in FIG. 1. The following nested PCR primers were then synthesized based on these protein sequences:

A) 5' GCAAGGTGTGACTTTTGTTC 3' (SEQ ID NO:10)

B) 5' GCGCTCGAGAGCATCACTCAG 3' (SEQ ID NO:13)

C) 5' GCGCGGCCGCAGTAAAATCCA 3' (SEQ ID NO:14)

D) 5' AGGATTTCTTCCCCTGTGTA 3' (SEQ ID NO:11)

The underlined portions of these oligonucleotides are the regions which hybridise to the flt cDNA sequence.

The other nucleotides were added to facilitate directional cloning. The cycles used were: first round with primers A and D [95.degree. C. 30 seconds, 55.degree. C. 30 seconds, 72.degree. C. 30 seconds].times.25; second round with primers B and C: [95.degree. C. 30 seconds, 44.degree. C. 30 seconds, 72.degree. C. 30 seconds].times.2 [95.degree. C. 30 seconds, 65.degree. C. 30 seconds, 72.degree. C. 30 seconds].times.25. The internal primers B and C had sites for the restriction enzymes Xho I and Eag I respectively at their 5' ends to permit directional cloning of the products.

It was found that certain tissues gave rise to PCR amplification products of notably larger size (as judged by agarose gel electrophoresis) than observed for the full length FLT cDNA product. Typical results are shown in FIG. 2.

PCR products obtained using the nested set of primers A-D were run out on a gel. Lanes 1-3 are products obtained from primary tissue samples of the ovarian carcinomas designated 17, 17R and 25R. Lanes 4 to 7 are products obtained from cell lines established from the ovarian carcinomas 7, 17R, 25 and 25R. Lanes 8 to 10 are the cell lines HEC 1-A, HEC 1-B and Ishikawa respectively. Lane 11 contains products from HUVECs.

The standard size band was of the expected size (around 285 bp) and was found to be identical to the 3' end of the published fit sequence (Shibuya et al., 1990 Oncogene 5, 519-524). However it can be clearly seen that in addition to the full length fit cDNA PCR-amplified product, in lanes 2 (17R, primary tissue) and 4 (7, cell line) are larger bands of approximately 360 bp. A faint band of similar size was also apparent in lane 5 (17R, cell line) but is not clearly seen when the gel photograph is reproduced. These larger bands were extracted from the gel by known techniques and subcloned into the plasmid vector pBluescript II KS and then subjected to sequence analysis using the dideoxynucleotide sequencing method (Sanger et al., 1977 PNAS 71, 5463-5467).

Sequencing of five independent clones (Boocock et al., 1995 J. Natl. Cancer Inst. 87, 506-516) revealed that each contained one of two novel insertions within the published fit sequence, in the region between the primers. Three of these clones (termed FLT5, FLT15 and FLT16) contained an 85 bp insertion at about position 1555, whilst two other clones (FLT13 & FLT14) contained a 65 bp insertion at about position 1665 (see FIG. 3, numbering based on that of Shibuya et al., 1990 cited above). The insertions account for the larger band size of the PCR products. However, both insertions contain an in-frame termination codon, so that corresponding full length RNAs would encode soluble, truncated receptor variants comprising the first five immunoglobulin-like domains of the extracellular region, up to amino acid 517 or 553, with either 24 or 14 (of which 13 are additional) unrelated amino acids at the C-terminus.

Although these variant fit clones were derived from partial cDNAs encoding only amino acids 503 onward, PCR products of the sizes predicted for corresponding full length cDNAs were amplified from cDNA derived from HUVEC cells, human chorion and ovarian carcinoma cell line 7, using primers specific for each of the novel insertions together with a primer binding just 5' of the initiating ATG (data not shown).

FIG. 4 is a schematic representation of various FLT receptor molecules. At the top, (a) shows the wild type, full length FLT receptor molecule, (b) represents the truncated version described by Kendall & Thomas, (c) represents the polypeptide encoded by FLT4 and (d) represents the polypeptide encoded by FLT15. The numerals at the right show the number of amino acids in the molecule and numerals in the boxes represent the number of amino acids present in the sFLT variants but not in the wild type molecule.

FIG. 5 shows the predicted C terminal amino acid sequence of the polypeptides which would be encoded by "full length" FLT4 and FLT15 clones (i.e. clones which contained all the nucleotide sequence 5' of the primer site used to generate the actual clones). The last 14 amino acids (SEQ ID NO:16) of the FLT4 clone, and the